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Phenotypic and Genetic Changes in Coxsackievirus B5 Following Repeated Passage in Mouse Pancreas In Vivo

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Common enterovirus infections appear to initiate or facilitate the pathogenetic processes leading to type 1 diabetes, and also sometimes precipitate the clinical disease. In experimental infection of mice, coxsackieviruses have shown to have a strong affinity for the exocrine tissue, while even in lethal cases, the islets remain unaffected. The virus strain most intensively studied in this respect is the diabetogenic variant E2 of coxsackievirus B4. In addition, it is known that all six serotypes of coxsackie B viruses can be made diabetogenic by repeated passages in either mouse pancreas in vivo or in cultured mouse beta-cells in vitro. However, the genetic determinants of the phenomenon have not been determined. In the present study, a laboratory strain of coxsackievirus B5 was passaged repeatedly in mouse pancreas in vivo. After 15 passages, the virus phenotype was clearly changed and infection of the variant resulted in a diabetes-like syndrome in mice characterized by chronic pancreatic inflammation together with dysregulation in glucose metabolism, loss of pancreatic acinar tissue, and mild insulitis. In order to characterize the genetic determinants involved in mouse pancreas adaptation, the passaged virus variant together with the parental virus strain was cloned for molecular characterization. The whole genome sequencing of both virus strains revealed only limited differences. Altogether, eight nucleotides were changed resulting in five amino acid substitutions, of which three were located in the capsid proteins. ***J. Med. Virol. 75:566–574, 2005.*** © 2005 Wiley-Liss, Inc.

KEY WORDS: enterovirus; coxsackievirus; mouse pancreas; repeated passages; chronic inflammation; increased blood glucose; genetic determinants

INTRODUCTION

Type 1 diabetes is a chronic disease characterized by specific destruction of the insulin producing β-cells in the Langerhans pancreatic islets. The pathogenetic process finally leading to complete β-cell loss and onset of clinical disease may start years before any clinical symptoms and this preclinical stage is characterized by appearance of β-cell autoantibodies in the circulation. In addition to the strong genetic component, environmental factors are invoked to explain the specific features of type 1 diabetes epidemiology. According to the results from previous cross-sectional and prospective studies on type 1 diabetes patients and/or prediabetic individuals, common enterovirus infections, probably together with other environmental factors, appear to initiate or facilitate the pathogenetic processes leading to type 1 diabetes, and also sometimes precipitate the clinical disease [Hiltunen et al., 1991, 1997; Clements et al., 1995; Hyoty et al., 1995; Andreoletti et al., 1997; Roivainen et al., 1998; Yin et al., 2002; Craig et al., 2003].

Enterovirus infection in man is usually initiated in the respiratory or gastrointestinal mucosa and spreads through the lymphatics to the circulation. After a brief viraemic phase, the virus reaches secondary replication sites in specific tissues and organs. Although it has been known for some time that coxsackie type B viruses are capable of infecting the human pancreas [Yoon et al., 1989], it has not been clear whether this is relevant to the development of type 1 diabetes. Recently, in situ

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hybridization studies on post-mortem pancreatic specimens of type 1 diabetic patients revealed enteroviral positive cells exclusively in the islets [Ylipaasto et al., 2004] suggesting that a direct virus-replication induced effect may be responsible for islet destruction.

There are two documented cases, where coxsackie B viruses have been isolated from children with acute-onset diabetes, and the virus isolates were shown to cause diabetes-like syndrome when injected into susceptible mice strains, CD1 and/or SJL [Yoon et al., 1978; Champsaur et al., 1980]. Although coxsackievirus-induced hyperglycaemia in mice is transient, persistence of the pathological process can be enhanced by re-infections with the same virus as shown recently by Flodstrom et al. [2003] and Horwitz et al. [2003]. All six prototype strains of coxsackie B viruses, which initially fail to produce diabetes in mice, can be made diabetogenic by repeated passages in either mouse pancreas in vivo or in cultured mouse β-cells in vitro [Toniolo et al., 1982]. The virus-induced diabetes is not restricted to mice since a variant of the prototype strain CBV-4/J.V.B., adapted to monkey β-cell, was reported to be capable of producing transient diabetes in non-human primates as well [Yoon et al., 1986]. However, the viral determinants responsible for adaptation-induced changes in the phenotype are not known.

In the present study, it is shown that fifteen successive passages of a parental CBV-5 strain in mouse pancreas resulted in apparent changes in the virus phenotype resulting in a capacity to induce chronic pancreatic inflammation together with dysregulation in glucose metabolism in CD-1 mice. The passaged variant together with the parental virus strain was cloned for molecular characterization and the genetic modifications responsible for the altered phenotype were determined.

MATERIALS AND METHODS

Virus

The parental coxsackievirus B5 strain DS (CBV5-DS-1) was plaque purified from a laboratory strain kindly provided by Dr. D. See. The identity of virus stock prepared by amplification of the plaque-purified virus was confirmed by a neutralization assay using type specific antisera. The known diabetogenic strain E2 of CBV-4, kindly obtained from Prof. J.W. Yoon, was used as a control in the first experiment.

Mice and Virus Passages In Vivo

Male CD-1 mice were obtained from Harlan, The Netherlands, and housed in a Macrolon M3 cage in a group of four animals. At the age of 5 weeks, the mice were infected intraperitoneally with CBV5-DS-1 (3 millions infectious units/per mice, 2 mice). At 3 days after infection, the mice were sacrificed and the first passage of the virus was obtained by extracting the pancreas (crushing the frozen tissue by slice). In order to obtain the second passage, a new set of mice were infected in a similar way with 200 μl of the first passage of the virus strain. The passages were continued up to 15 passages (2–12 mice/passage) and the final harvest designated as CBV5-DS-1-MPP.

Mice Infection Experiments

Parallel groups of mice were infected via the intraperitoneal route with the parental virus strain, CBV-5-DS-1 (0.3 million infectious units/mouse), or with about the same infectious dose of mouse pancreas-passaged virus, CBV5-DS-1-MPP (0.4 million infectious units/mouse). In the latter case, virus dilution corresponding to 0.1 million infectious units was also used for mice infections. Mice were killed on selected days after infection and tissues (pancreas, spleen, heart) and blood were collected. One-half of the tissue sample was frozen for virus titration and the other half was fixed with 4% formaldedyde for histological staining and immunohistochemistry. All studies on mice were approved by the Institutional Committee on Animal Care and Use, with the final permission granted by the Provincial Government of Southern Finland.

Blood Glucose Measurements

At various times post-infection, the fasting mice were inoculated intraperitoneally by 2 mg glucose per gram body weight, 60 min later glucose levels were measured from venous blood by using the MediSense Precision Plus Electrodes, Abbott Laboratories, UK. The mean level of blood glucose of 32 uninfected mice was 8.5 mmol/l ± 1.9. Mice with glucose levels more than 3 SD above the mean were considered to have dysregulation in glucose metabolism.

Histological Stainings

Mice were killed at various times post-infection and paraffin sections of formalin fixed pancreas were prepared, cut into 5-μm thick sections and stained with hematoxylin and eosin (H&E) or with the primary virus specific polyclonal antiserum (1:2,000). In the latter case, slides were counterstained in Mayer's hematoxylin. In immunochemistry, the primary antibody was detected with peroxidase-conjugated secondary antibody (Vector, Burlingame, CA).

Scoring of Morphological Changes in Pancreatic Tissues

Pancreatic sections were stained with H&E and ranked for morphological changes with the following criteria. Codes: 0, no histopathological changes; AI, acute inflammation in exocrine tissue; CI, chronic inflammation in exocrine tissue; N, necrosis in exocrine tissue; A/F, atrophy, fibrosis in exocrine tissue; L, inflammation in islets. Grades: +, very mild; ++, moderate; +++, severe.

Cloning of CBV5-DS-1 and CBV5-DS-1-MPP

Total RNA was extracted from cells infected with CBV5-DS-1 and CBV5-DS-1-MPP (14 passage) using

TABLE I. Primers Used for Cloning of Viral Genomes

Forward primers:
EP1, 5′-GAG ATC GAT TAA TAC GAC TCA CTA TAG GTT AAA ACA GCC TGT GGG-3′
EP3, 5′-GTA CTA CCA CAG CTC ATG GG-3′
Reverse primers:
EP2, 5′-GGG ACT GGT ACC TCT TAG GG-3′
EP4A, 5′-CCG CGG CCG CTT TTT TTT TTT TTT TTT TTT TCC GCA CCG AAT-3′

Trizol Reagent (Gibco BRL, Life Technologies, Gaithersburg, MD) and used as a template for first-strand synthesis of cDNA (SuperScript First-Strand Synthesis System for RT-PCR Kit, Gibco BRL, Life Technologies, Gaithersburg, MD) by using reverse primer EP4. Two primer pairs (EP1 and EP2; EP3 and EP4, Table I) were used to amplify 3605 and 3922 nucleotide-long PCR products, representing two partially overlapping halves of the full-length virus genomes (nt 1-3584 and 3477–7399). The amplification was done using SuperScript One-Step RT-PCR for Long Templates Kit (Gibco BRL, Life Technologies). Purified PCR products of both halves were cloned into pGEM-T Easy Vector System I Kit (Promega, Madison, WI) and the clones were transfected into competent bacterial cells (*Escherichia coli* DH5α). Plasmid DNA was extracted from bacteria using QIAprep Spin Miniprep Kit Protocol, QIAGEN, Hilden, Germany.

PCR-Sequencing

The viral clones were sequenced using the primer walking strategy. The cycle sequencing reactions were performed using ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Warrington, UK). Automated sequencing equipment ABI Prism 310 Genetic Analyzer was used. The designed oligonucleotides used as primers (Table II) were purchased from DNA Technology A/S (Aarhus, Denmark). The sequence data were analyzed using Vector NTI Suite 6 program.

TABLE II. Primers Used for Sequencing of Virus Genomes

Primer sequence	Primer name	Location of sequencing primer
5′-GAG ATC GAT TAA TAC GAC TCA CTA TAG GTT AAA ACA GCC TGT GGG TTG-3′	EP1	T7 + Cla1 and 1-20 the last 20 nucleotides)
5′-GGG ACT GGT ACC TCT TAG GG-3′	EP2	3565–3584
5′-GTA CTA CCA CAG CTC ATG GG-3′	EP3	3423–3442
5′-CCG CGG CCG CTT TTT TTT TTT TTT TTT TTT TCC GCA CCG AAT-3′	EP4A	7390-7400 (the first 10 nucleotides and Poly (A) -tail)
5′-CCG TGA TAC CAG ATG CTG GCG C-3′	HP1e	44–66
5′-ATC AGG TCG ATG AGT CAC CGC A-3′	HP2	315–336
5′-CGA ACT GTG CTC ACA ATG GGG C-3′	HP3	3132–3153
5′-GTG TCG ATG CAC TAC CGG AGT G-3′	HP4	3460–3481
5′-CGT GTA TGT CTT TCA TGG GC-3′	HP5	7090–7109
5′-GCT CTG TTG GAT ACC GGA TG-3′	HP6	636–655
5′-TTG GAT ATG GAG TGT GGC-3′	HP7	1047–1064
5′-TCG GAT TGG TGG ACG TCT GC-3′	HP8	2929–2948
5′-CAG GCA TGG GAA TCG GTG TGG G-3′	HP9	1467–1488
5′-TTC AGC AGC GGT CAA GGC GGG A-3′	HP10	2530–2551
5′-TTT CCG AGC CAG GCG ACT GTG G-3′	HP11e	3606–3627
5′-CCC AGT TTG ATG TCA CGC CC-3′	HP12	1809–1828
5′-GCG GGG TGG CAT GCC TTC AGG-3′	HP13	6760–6780
5′-CGC ATC TAT GGG CCA CGG G-3′	HP14	6916–6934
5′-GGG TGG TGA AGG TGT GGT GGG G-3′	HP15	3673–3694
5′-GCA CCG CGT CAC CGT GGC GGT GG-3′	HP16	3969–3991
5′-GGC AGG CAA ACC AGA CGG GGC TC-3′	HP17	6634–6656
5′-GCG CTC CGT CCC AGA GTG ACC-3′	HP18	4239–4259
5′-GCC CAA TGC AAC ATA AGG GTA GC-3′	HP19	6261–6283
5′-CCC GAT GGG AAG GAT GTG TCC-3′	HP20	4586–4606
5′-CCT TGA GGC GTG GGT CAC C-3′	HP21	6047–6065
5′-GGG TCC TCC AGT GTC AGG G-3′	HP22	5029–5048
5′-CGT TAA CTT CAC CTC CTC CTT GGC-3′	HP23	5633–5657
5′-GCT TGA TCA AGA CAT GGC TGG-3′	HP24	151–171
5′-GGC CAT CCG GTA TCC AAC AG-3′	HP25	633–652
5′-GGA GCG TTC CAG TTG GAC G-3′	HP26	7224–7242
5′-GGA TGC GTT GCA TTG CAC G-3′	THP27	1276–1294
5′-CGG TGT ATT CGT CCA TCA CC-3′	THP28	2269–2288
5′-CCT TGA CCG CTG CTG AAA CG-3′	THP29	2535–2554
5′-CGT GGG TCC CAC GAC CAC C-3′	THP30	3183–3201
5′-CCT CAT AGG TTG CAC CGC GTC-3′	THP31	3958–3978
5′-GCT CCT ACA CGG GAG TCC TGG-3′	THP32	4417–4437
5′-CCA CAC TGG ACA TTA GCA CC-3′	THP33	6168–6187
5′-CCG CTA GTG TGT GGA AAG GCC-3′	THP34	4889–4909
5′-GGG TAC CTT GGG CTT CTG G-3′	THP35	5320–5338

Statistical Analyses

Differences between different groups of mice were compared by using two-tailed Mann–Whitney U-test.

RESULTS

Tropism of CBV5-DS-1 to Mouse Pancreas

Parallel groups of male CD-1 mice were infected with CBV5-DS-1 or CBV-4E2. At different time points after infection, the mice were killed and the pancreas, heart, and spleen were assayed for total infectivity. At 3 days after infection, high virus titers were found in pancreatic tissues of CBV5-DS-1 infected mice, while the concentration of virus in other organs remained low (3–4 log lower) as shown in Figure 1. In contrast, CBV-4-E2 was found distributed in mouse spleen and heart in addition to pancreas. In both cases, infectious viruses were no longer found in tissue samples collected at 1 week after inoculation. When two other mouse strains, NIH and SJL, were tested for CBV5-DS-1 infection, practically similar results were obtained (not shown), demonstrating that CBV-5-DS-1 has a predilection for pancreatic cells.

Repeated Passaging of CBV-5-DS-1 in Mouse Pancreas In Vivo

Due to its propensity for growth in the pancreas (Fig. 1), CBV-5-DS-1 was selected as a parental virus for further passaging. CBV-5-DS-1 was passaged repeatedly for 15 times in mouse pancreas in vivo (CBV5-DS-1-MPP). Although virus titres in pancreatic tissues increased during repeated passages, they also increased in other organs (Fig. 2). In order to gain better understanding of the effect of virus replication in vivo, parallel groups of mice were infected intraperitoneally with CBV5-DS-1-MPP or the parental CBV5-DS-1 virus, and the infection-induced consequences at various time points were compared between the groups.

Histological examination of tissues showed that soon after infection (at 2–3 days), both CBV5-DS-1 and CBV5-DS-1-MPP viruses caused acute pancreatic damage (Figs. 3 and 4). The histological scoring by a pathologist showed that both groups of infected mice had acute inflammation in the acinar tissue and mild inflammation in the islets of Langerhans at 3 days after infection (Fig. 4). The signs of necrosis in the acinar tissue were more prominent in mice infected with the virus strain that had been passaged in vivo than in those infected with the parental strain, CBV5-DS-1 (Fig. 4). Immunohistochemistry to detect viral antigens revealed that in addition to the acinar tissue, the passaged virus strain can be occasionally found in the islets of Langerhans at 3 days after infection (Fig. 5B,C).

In the case of the parental virus strain, the damage to the acinar tissue was highly restricted and the majority of the exocrine pancreas remained unaffected. In addition, all signs of pancreatic infection induced by CBV-5-DS-1 had disappeared by day 3 p.i. (Fig. 3, in other experiments right after that). In contrast, the destructive effect of the CBV5-DS-1-MPP virus was evident for much longer. As shown in Figure 4 signs of moderate atrophy/fibrosis and mild chronic inflammation were found in the acinar tissue at 6 weeks after infection (Fig. 4). Furthermore, signs of mild inflammation were seen at long period of time in islets (Figs. 4 and 6).

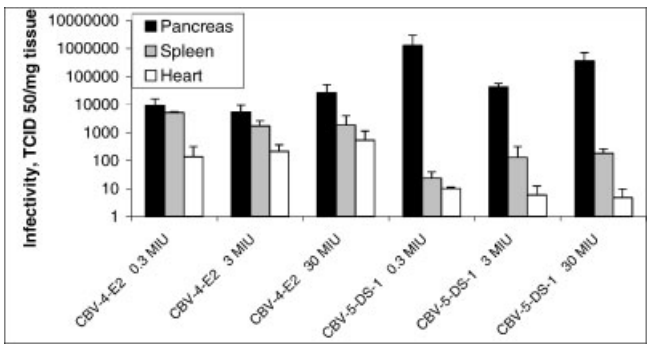

Fig. 1. Infectivities of CBV-5-DS-1 and CBV-4-E2 in different tissues of mice at 3 days after infection. Two parallel mice were infected with CBV-5-DS-1 (0.3–30 million infectious units (MIU)/mouse) or the known diabetogenic strain E2 of CBV-4 (0.3–30 million infectious units (MIU)/mouse). Three days after infection, the mice were sacrificed and one-half of tissues were collected for infectivity measurements. Results in the figure are shown as means + SD.

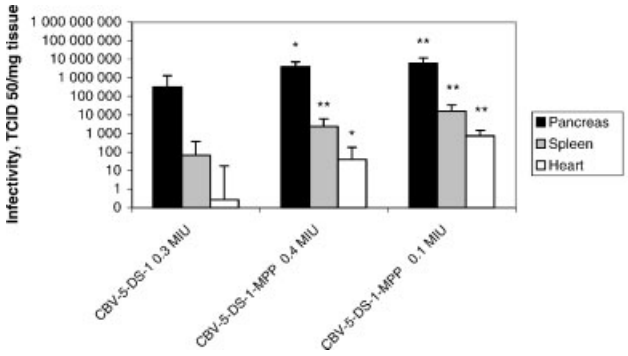

Fig. 2. Infectivities of mouse pancreas in vivo passaged virus strain in different tissues of mice at 3 days after infection. Parallel groups of mice (16 in each group) were infected with about equal infectious doses of the parental virus, CBV-5-DS-1 (0.3 million infectious units (MIU)/mouse) or the mouse pancreas in vivo passaged virus strain, CBV-5-DS-1-MPP (0.4 million infectious units (MIU)/mouse). In the latter case also, dilution corresponding to 0.1 million infectious units (MIU) was used for mice infections. Three days after infection, five mice from each group were sacrificed and one-half of tissues were collected for infectivity measurements. Results in the figure are shown as medians + SD. The rest of animals were used for blood glucose measurements and histological analyses. Significant differences in infectivities of tissues between the groups of mice infected with the parental virus, CBV-5-DS-1 or CBV-5-DS-1-MPP are shown in the figure. $^{*}P < 0.05$; $^{**}P < 0.01$.

Virus Strain Repeatedly Passaged in Mouse-Pancreas In Vivo Causes Chronic Pancreatic Inflammation

During the repeated passages in vivo, the capability of the virus strain CBV-5-DS-1 to cause chronic inflammation of the pancreas increased (not shown). In

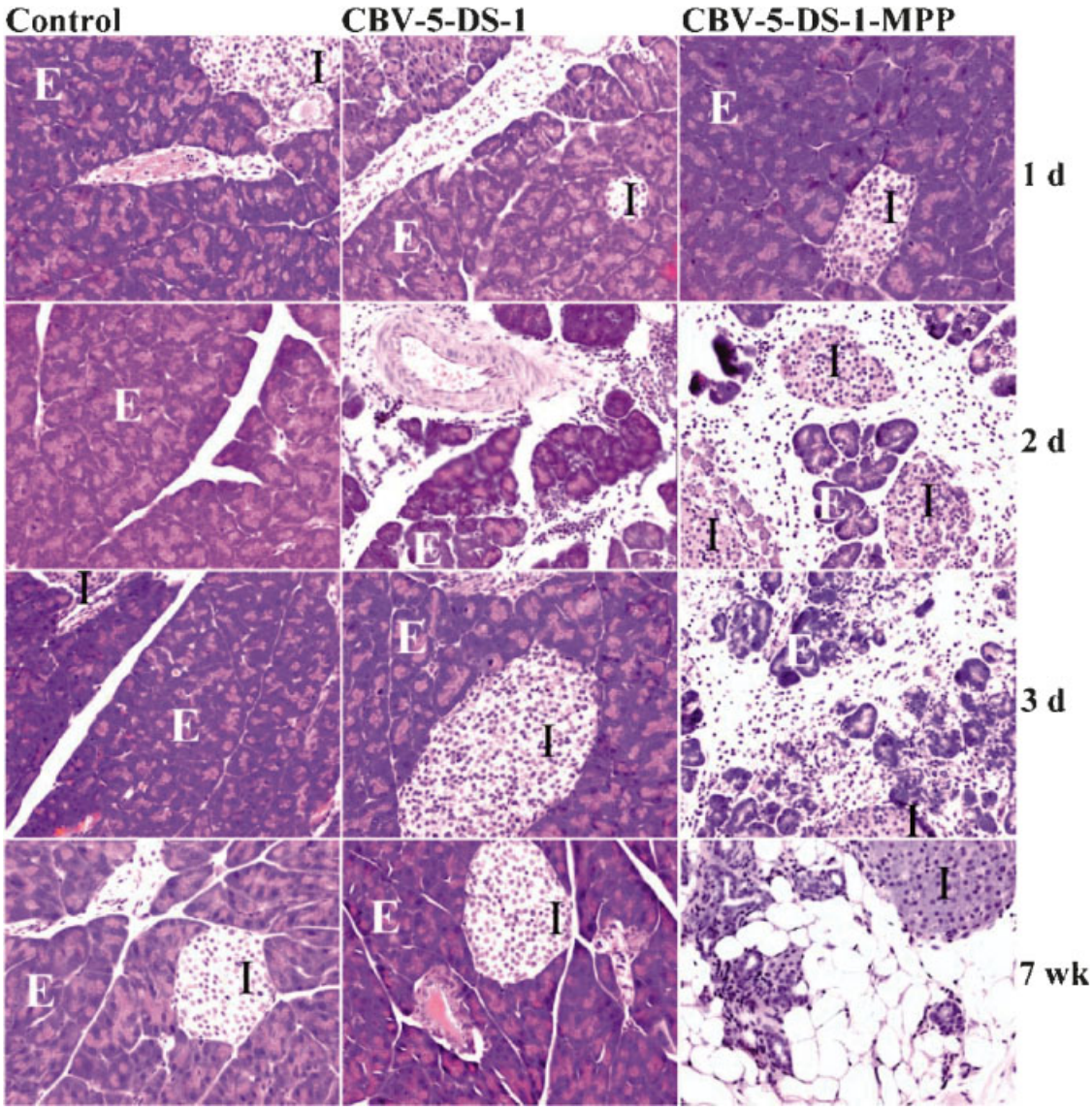

Fig. 3. Infection induced histological changes in mouse pancreas. Parallel groups of mice were infected with the parental virus, CBV-5-DS-1 or the mouse pancreas in vivo passaged virus strain, CBV-5-DS-1-MPP. At various times after infection, subgroups of mice were sacrificed for histological examination of pancreatic tissue. Formaldehyde fixed paraffin blocks were cut into 5-μm sections and stained with hematoxylen and eosin. One representative sample from each group is shown in the figure. Islets and exocrine tissue are indicated by alphabets I and E, respectively.

experiments where the virus-induced histopathological changes in the pancreas were examined over a longer period of time, signs of both atrophy/fibrosis and chronic inflammation in the acinar tissue were still found at 9 and 15 weeks after infection (Fig. 7). Furthermore, mild inflammation was evident in islets (Fig. 7). It should be emphasized here that at these later time points, all pancreatic tissues from mice infected with the parental virus were identical to those of uninfected animals.

The groups of infected mice were also followed for glucose metabolism by monitoring blood glucose levels in mice 60 min after the glucose inoculation. As shown in Figure 8, the glucose levels were slightly but significantly increased in mice infected with the parental virus strain at 3 weeks after infection. Mice infected with the mouse pancreas passaged virus strain showed definitely higher median levels at 3 weeks after infection (Fig. 8), suggesting that this virus infection was capable of affecting the islets of Langerhans.

Genetic Characteristics of Mouse Pancreas Passaged Virus Strain, CBV5-DS-1-MPP

In order to determine genetic characteristics relevant to the altered phenotype both virus genomes, the mouse-pancreas passaged strain CBV5-DS-1-MPP and

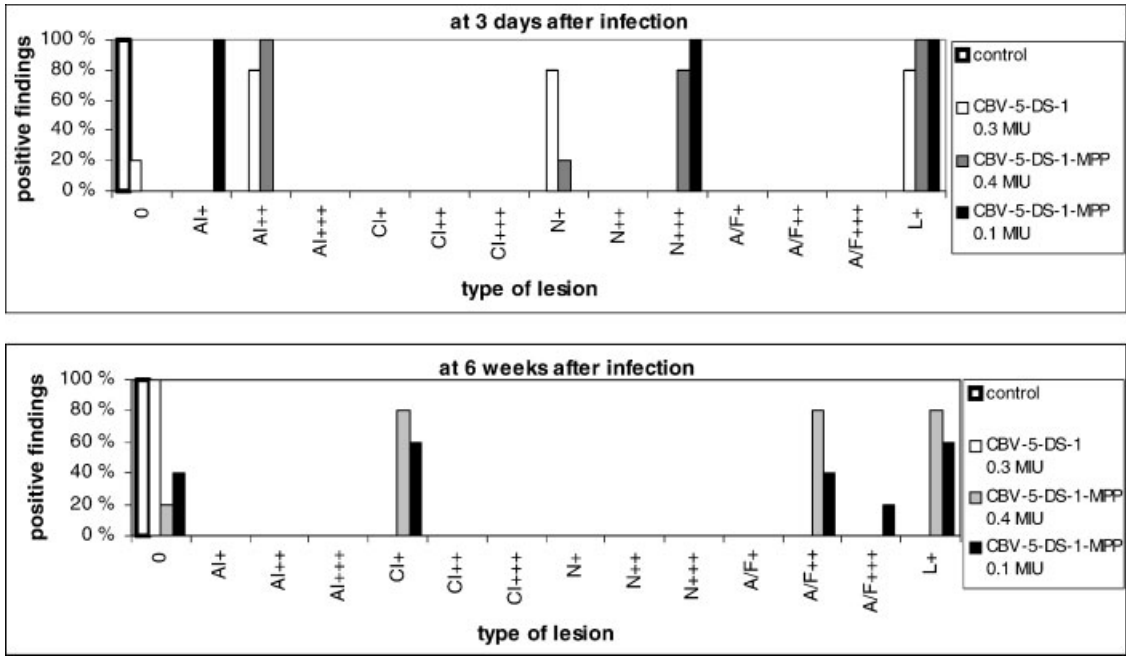

Fig. 4. Infection induced histological changes of pancreas. Pancreatic tissue was collected from mice sacrificed at 3 days and 6 weeks post-infection (five mice from each group at each time point) and fixed with 4% formaldehyde. In histochemistry, the paraffin sections were stained with hematoxylen and eosin and ranked for morphological changes. Codes: 0, no histopathological changes; AI, acute inflammation in exocrine tissue; CI, chronic inflammation in exocrine tissue; N, necrosis in exocrine tissue; A/F, atrophy, fibrosis in exocrine tissue; L, inflammation in islets. Grades: +, very mild; ++, moderate; +++, severe.

its parental strain CBV5-DS-1 were subjected to PCR-cloning and nucleotide sequencing. First, the complete virus genome was cloned in two partially overlapping halves and then sequenced by using the primer walking strategy. Comparison of the entire genomic sequences revealed only limited differences between the two virus strains (Table III). Altogether, eight nucleotides appeared to have changed during repeated passage of the virus in mouse pancreas in vivo resulting in only five amino acid substitutions. Three amino acid changes were located in the capsid region including VP4 (M47T), VP3 (R129K), and VP1 (S73N) while two amino acid changes were found in non-structural viral proteins, in particular 2C (D261N) and VPg (V47L).

DISCUSSION

Adaptation of an enterovirus strain to enhance replication in the secondary target tissues may significantly extend the cell tropism of a virus also affecting organ specific symptoms of infection. In the present study, we have (1) passaged the virus strain CBV5-DS-1 repeatedly in mouse pancreas in vivo, (2) characterized the virus strain, and (3) determined genetic characteristics of the altered phenotype.

The specific virus strain, CBV5-DS-1, was selected for our study, since it already showed a propensity to infect mouse pancreas. Fifteen successive passages of the virus through mouse pancreas resulted in apparent changes in phenotype. According to our results, the in vivo passaged virus strain was capable of causing so called diabetes-like syndrome in mice. In this study, the diabetes-like syndrome was characterized by chronic pancreatic inflammation together with mild glucose dysregulation, loss of pancreatic acinar tissue, and mild insulitis.

In experimental infections of mice, enteroviruses have shown to have a predeliction for the exocrine compartment while even in lethal cases the islets, ducts, and connective tissue remain unaffected [Ross et al., 1974; Szopa et al., 1989; Vuorinen et al., 1989; Vella et al., 1992; Arola et al., 1995; See and Tilles, 1995; Klingel et al., 1996; Ramsingh et al., 1997; Flodstrom et al., 2002a]. The reasons for the selected protection of islets are not yet entirely understood. Although the expression of HCAR, a cell surface receptor for all serotypes of coxsackie B viruses, has been reported to occur in human islets [Chehadeh et al., 2000; Ylipaasto et al., 2004], practically no evidence of the expression of the corresponding mouse protein has been found in the islets of murine pancreas [Mena et al., 2000]. In addition, according to Flodstrom et al. [2002a,b], β-cell response to interferons may protect islets from CBV-4 infection.

When distribution of the virus in mouse pancreas was studied by immunohistochemistry, the acinar tissue was found to be affected by both the parental strain CBV5-DS-1 and the mouse pancreas passaged strain, CBV5-DS-1-MPP. However, in the case of CBV5-DS-1-MPP, some staining was found in the islets of Langer-

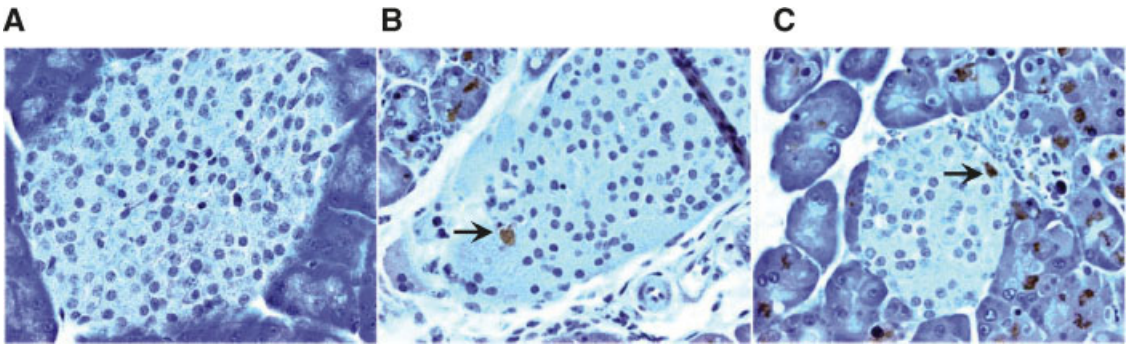

Fig. 5. Islet cells were infected with mouse pancreas in vivo passaged virus strain, CBV-5-DS-1-MPP. Three days after infection, mice were sacrificed and pancreatic tissues collected for immunohistological staining. Immunohistology with virus specific antiserum revealed few virus positive cells in islets (shown by arrows). **Panel A**, negative control; **panels B** and **C**, infected mouse.

hans as well. Another indication of an alteration of the in vivo passaged virus strain in its ability to infect β-cells derives from glucose measurements. Mice infected with CBV5-DS-1-MPP had higher glucose levels following delivering of a glucose bolus than mice either infected with the parental virus strain or left uninfected. However, the ability to normalize glucose levels of the infected mice varied from one time point to another. Another fact confusing the results further was that only subgroups of infected mice became hyperglycaemic. Previously, both characteristics have been described for the known diabetogenic strain E2 of CBV-4 [Toniolo et al., 1982; Horwitz et al., 2003; Yap et al., 2003].

The most prominent phenotypic feature of the mouse pancreas passaged virus strain, CBV5-DS-1-MPP, was its capability to induce chronic inflammation in pancreas. Even at 15 weeks post-infection, mice infected with this virus strain showed signs of inflammation of the acinar/exocrine tissue of the pancreas but also mild inflammation in islets of Langerhans. In contrast, pancreatic tissues from mice infected with the parental virus had recovered completely by 1 week after infection. This is similar to the report by See and Tilles [1995] that the diabetogenic isolate E2 of CBV-4 is capable of inducing chronic inflammation in mouse pancreas. Recently, Yap et al. [2003] described that a lack of islet neogenesis plays a role in β-cell depletion in mice infected with a diabetogenic variant E2 of CBV-4 while a remarkable regeneration takes place in exocrine pancreas of mice infected with prototype strain of CBV-4.

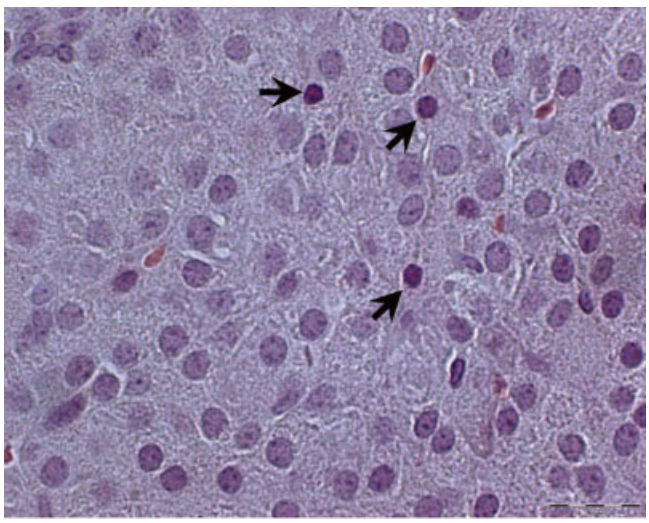

Fig. 6. Histological evidence of mild inflammation in islets. Mice infected with the mouse pancreas in vivo passaged virus strain, CBV-5-DS-1-MPP (0.4 million infectious units), showed signs of mild inflammation in islets at 6 weeks after infection.

There are several published examples showing that few point mutations or even a single one may lead to important changes in the biology of various picornaviruses [Bae and Yoon, 1993; Caggana et al., 1993; Knowlton et al., 1996; Jun et al., 1997; Schmidtke et al., 2000]. Only one residue, Thr-129 of VP1, is a major virulence determinant of CBV-4 replication in the mice acinar pancreas as shown by Caggana et al. [1993]. According to our hypothesis, viral genetic determinants for tropism for pancreas and/or to the cells of islets of Langerhans might be restricted to only one or few amino acids. Therefore, the two virus strains, the parental strain CBV5-DS-1 and the mouse pancreas in vivo passaged strain CBV5-DS-1-MPP, were cloned molecularly and subjected to sequencing. Comparison of the entire genetic sequences revealed only limited differences. Eight out of 7,400 nucleotides in the viral genome were changed resulting in five amino acid substitutions. Three of the changed amino acids are located in the capsid proteins while two of them are in the nonstructural proteins. In the three-dimensional atomic model of a protomer, based on the X-ray crystallographic analysis of CBV-3 [Muckelbauer et al., 1995], two out of the mutated amino acids in the capsid proteins are located at or close to the outer surface of the virion suggesting that they might be involved in virus-cell interactions possibly augmenting initiation of virus infection. On the other hand, mutations in the nonstructural genes may also affect virulence. In further studies, the availability of molecular clones of both virus

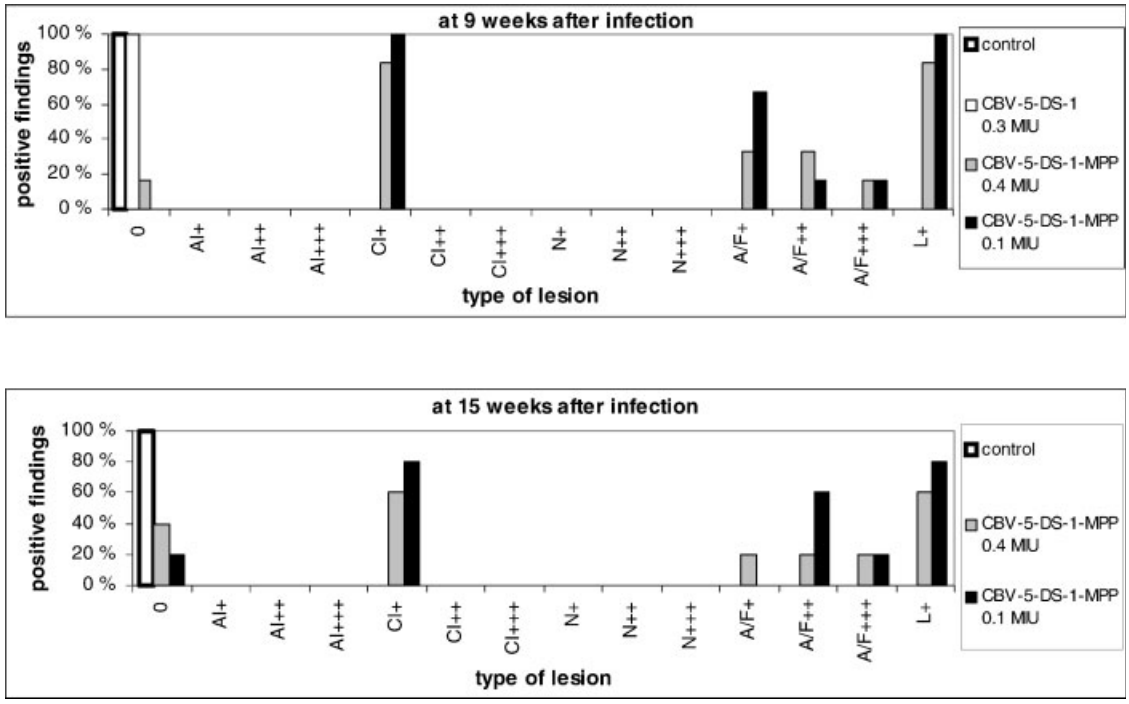

Fig. 7. Histological evidence of chronic inflammation caused by the mouse pancreas in vivo passaged virus strain, CBV-5-DS-1-MPP. Pancreatic tissues were collected from mice sacrificed at 9 and 15 weeks post-infection (five mice from each group at each time point) and fixed with 4% formaldehyde. In histochemistry, the sections were stained with hematoxylen and eosin and ranked for morphological changes. Codes: 0, no histopathological changes; AI, acute inflammation in exocrine tissue; CI, chronic inflammation in exocrine tissue; N, necrosis in exocrine tissue; A/F, atrophy, fibrosis in exocrine tissue; L, inflammation in islets. Grades: +, very mild; ++, moderate; +++, severe.

strains will allow construction of recombinants to assess the role of different genomic domains. Subsequently, the precise genetic determinants of the phenotypic features, determing β-cell tropism and cytopathology, could be identified by site-directed mutagenesis in a study that is in progress.

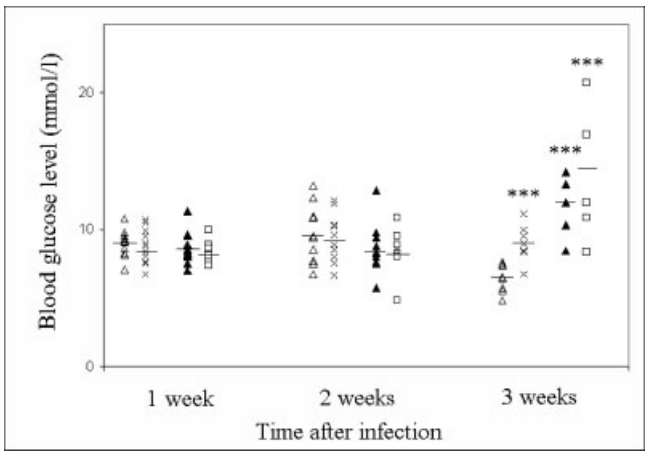

Fig. 8. Blood glucose levels were increased in mice infected with the mouse pancreas in vivo passaged virus strain. At 1–3-week post-infection, fasting mice (11–13 mice/group) were inoculated with glucose and 60 min later the glucose levels were measured from vein blood. The median value in each group is marked with a line. Symbols: △, uninfected control; × CBV-5-DS-1 0.3 MIU; ▲, CBV-5-DS-1-MPP 0.4 MIU; □, CBV-5-DS-1-MPP 0.1MIU. Significant differences between uninfected and infected groups of mice are shown in the figure, $^{***}P < 0.005$.

TABLE III. Nucleotide and Amino Acid Changes Induced by Repeated Passaging of CBV-5-DS-1 in Mouse Pancreas In Vivo

		CBV-5-DS-1-MPP		CBV-5-DS-1	
Protein	Position no.	NT	AA	NT	AA
VP4					
nt	138	T	M	C	T
aa	47				
VP2					
nt	268	G	G	A	G
aa	89				
VP3					
nt	384	G	R	A	K
aa	129				
VP1					
nt	282	G	S	A	N
aa	95				
2A					
nt	73	C	H	T	H
aa	24				
2C					
nt	779	G	D	A	N
aa	261				
Vpg					
nt	140	G	V	C	L
aa	47				
3C					
nt	415	C	T	T	T
aa	139				

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